

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> C07H 21/04, C07K 14/00, 14/195, 14/435, 17/00, 17/06, 17/14, C12N 1/21, 15/00, 15/09, 15/63, 15/70, C12P 21/00, 21/02, G01N 33/53, 33/547, 33/566, A23J 1/00	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/10253</b>  <b>(43) International Publication Date:</b> 20 March 1997 (20.03.97)
<b>(21) International Application Number:</b> PCT/US96/14567 <b>(22) International Filing Date:</b> 11 September 1996 (11.09.96)  <b>(30) Priority Data:</b> 60/003,819                      15 September 1995 (15.09.95)    US 9605210.5                      12 March 1996 (12.03.96)        GB  <b>(71) Applicant (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MARCY, Alice [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). SA-LOWE, Scott, P. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). WISNIEWSKI, Douglas [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(74) Common Representative:</b> MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).	<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS  <b>(57) Abstract</b>  This application describes a high throughput assay for screening for compounds capable of binding to a fusion protein which consists of a target protein and an FK506-binding protein. The method for preparing the DNA encoding for the fusion protein and for expressing that DNA is also described in the application. The invention also discloses the recombinant DNA and protein sequences for several fusion proteins.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

- 1 -

**TITLE OF THE INVENTION****A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS****BACKGROUND OF THE INVENTION**

5               Src homology 2 (SH2) domains are a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. They have routinely been expressed in *E. coli* as fusion proteins with glutathione-S-transferase (GST). This usually provides high level  
10 expression and straightforward affinity purification on glutathione-Sepharose. Ligand binding is then assayed by incubating the GST/SH2 with a radiolabeled phosphopeptide, precipitating the complex with glutathione-Sepharose, washing the beads, and then counting the beads to determine bound radioactivity [Isakov et al., *J. Exp. Med.*, 181, 375-  
15 380 (1995); Piccione et al., *Biochemistry*, 32, 3197-3202 (1993); Huyer et al., *Biochemistry*, 34, 1040-1049 (1995)]. There are several disadvantages to this procedure, particularly when applied to high-throughput screening for agonists, antagonists, or inhibitors as new leads for drug development. First, the radiolabeling of the peptide is  
20 carried out either enzymatically with a kinase and [<sup>32</sup>P]ATP or chemically with [<sup>125</sup>I]Bolton-Hunter reagent. In both cases, the isotopes are short-lived and thus require frequent preparation of material. In the case of enzymatic phosphorylation, the appropriate kinase must also be available in sufficient quantity to generate enough  
25 material for screening purposes. Second, the protocol requires separation of bound complex from free phosphopeptide by washing of the glutathione-Sepharose beads. This is a nonequilibrium procedure that risks dissociation of the bound ligand, particularly when off-rates are fast. Thus, there is the possibility of misleading results. Finally,  
30 due to the number of manipulations and centrifugations involved, the protocol is very tedious to conduct manually and is not readily adaptable to robotic automation to increase throughput.

Two additional methods for measuring the interaction of proteins and ligands that have been applied to SH2 domains are

- 2 -

biospecific interaction analysis using surface plasmon resonance and isothermal titration calorimetry (Felder et al., *Mol. Cell. Biol.*, 13, 1449-1455 (1993); Panayotou et al., *Mol. Cell. Biol.*, 13, 3567-3576 (1993); Payne et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90, 4902-4906 (1993); Morelock et al., *J. Med. Chem.* 38, 1309-18 (1995); Ladbury et al., *Proc. Natl. Acad. Sci. U.S.A.*, 92, 3199-3203 (1995); Lemmon et al., *Biochemistry*, 33, 5070-5076 (1994)). These techniques do not require a particular fusion partner for the SH2 domain, but do require sophisticated instrumentation that is not amenable to high throughput screening.

#### SUMMARY OF THE INVENTION

The instant invention covers a method of screening for compounds capable of binding to a fusion protein which comprises combining a test compound, a tagged ligand, a fusion protein (target protein, peptide linker and FK506-binding protein), and a radiolabeled ligand in a coated microscintillation plate, and then measuring the scintillation counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay in the absence of the test compound, so as to determine the effect the test compound has on the binding of the tagged ligand. Also within the scope of this invention are the processes for preparing and expressing the recombinant DNA encoding a fusion protein. This invention further relates to the recombinant DNA expression vector capable of expressing the fusion protein. This invention further relates to a process for purifying the recombinant fusion protein. This invention provides an immediate means of making use of microscintillation plate technology for the functional assay of ligand binding to a single or multiple signal transduction domain(s), for example a phosphopeptide binding to an SH2 domain. The present invention does not require specialized radiochemical synthesis and is readily adaptable to robotic automation for high capacity screening for agonists, antagonists, and/or inhibitors.

- 3 -

### BRIEF DESCRIPTION OF THE FIGURES

Figure 1.

- 5 A.) Binding of the streptavidin microscintillation plate, biotinylated ligand and the fusion protein (SH2:FKBP), which emits a detectable signal; and
- B.) Binding of the test compound and the fusion protein (SH2:FKBP), which results in no signal detection .

### DETAILED DESCRIPTION OF THE INVENTION

10 The present invention relates to a method of screening for compounds which preferentially bind to a target protein.

An embodiment of this invention is a method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

- 15 a) mixing a test compound, a tagged ligand, the fusion protein, and a radiolabeled ligand;
- b) adding the mixture to a coated microscintillation plate;
- c) incubating the mixture for between about 1 hour and about 24 hours;
- 20 d) measuring the plate-bound counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound using scintillation counting; and
- e) determining the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.
- 25

A second embodiment of this invention is a process for preparing a recombinant DNA expression vector encoding for a fusion protein comprising the steps of:

- 30 a) removing the stop codon on DNA encoding for an FK506-binding protein;
- b) synthesizing a modified DNA fragment on the DNA encoding for the FK506-binding protein which encodes for a peptide linker;

- 4 -

- 5
- c) digesting an expression vector at cloning sites;
  - d) cloning the modified DNA fragment encoding for the FK506-binding protein with a peptide linker into the digested expression vector to generate a recombinant DNA expression vector encoding for FK506-binding protein with a peptide linker; and
  - 10 e) cloning DNA encoding for a target protein into a recombinant DNA expression vector encoding for FK506-binding protein with a peptide linker to produce the recombinant DNA expression vector encoding for the fusion protein.

A third embodiment of this invention is a process for expressing recombinant DNA encoding for a fusion protein in an expression vector comprising the steps of:

- 15
- a) transforming a host cell with the fusion protein expression vector;
  - b) inducing expression of the fusion protein in the host cell;
  - c) recovering the fusion protein from the host cell; and
  - 20 d) purifying the fusion protein.

A fourth embodiment of this invention is a process for purifying an isolated FKBP-SH2 fusion protein, comprising the steps of:

- 25
- a) preparing an affinity matrix consisting of biotinylated phosphopeptide coupled to avidin or streptavidin immobilized on a solid support;
  - b) preparing a freeze/thaw extract from cells expressing the fusion protein;
  - c) loading the extract onto the affinity matrix and washing off unbound protein; and
  - 30 d) eluting the desired fusion protein with phenyl phosphate.

The term "fusion protein" refers to a "target protein" fused to an "FK506-binding protein" (FKBP), the two proteins being separated by a "peptide linker".

- 5 -

A "peptide linker" may consist of a sequence containing from about 1 to about 20 amino acids, which may or may not include the sequence for a protease cleavage site. An example of a peptide linker which is a protease cleavage site is represented by the amino acid  
5 sequence GLPRGS.

The term "target protein" refers to any protein that has a defined ligand. Included within this definition of target protein are single and multiple signal transduction domains, such as, but not limited to, Src homology 1 (SH1), Src homology 2 (SH2), Src homology 3  
10 (SH3), and pleckstrin homology (PH) domains [Hanks & Hunter, *FASEB J.*, 9, 576-596 (1995); Bolen, *Curr. Opin. Immunol.*, 7, 306-311 (1995); Kuriyan & Cowburn, *Curr. Opin. Struct. Biol.*, 3, 828-837 (1993); Cohen et al., *Cell*, 80, 237-248 (1995)]. The term "SH1 domain" refers to a family of homologous protein domains that bind  
15 ATP and catalyze tyrosine phosphorylation of peptide and protein substrates. The term "SH2 domain" refers to a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. The term "SH3 domain" refers to a family of homologous protein domains that  
20 share the common property of recognizing polyproline type II helices. The term "PH domain" refers to a family of homologous protein domains that mediate both protein-protein and protein-lipid interactions. Examples of SH2 domains which may be utilized in the method of the invention include, but are not limited to, the single and tandem SH2  
25 domains present in the tyrosine kinases ZAP, SYK and LCK. The DNA sequences were obtained from GenBank, National Center for Biotechnology Information, National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894. The Accession Numbers for the sequences are: human ZAP (L05148); human SYK (L28824) and human  
30 LCK (X13529).

The term "tagged ligand" refers to a biotinylated or epitope tagged ligand for the target protein.

The term "radiolabeled ligand" refers to a [ $^3\text{H}$ ]-, [ $^{125}\text{I}$ ]-, [ $^{14}\text{C}$ ]-, [ $^{35}\text{S}$ ]-, [ $^{32}\text{P}$ ]-, or [ $^{33}\text{P}$ ]-labeled ligand which binds to the FKBP.

- 6 -

An example of a radiolabeled ligand useful in the instant invention is [<sup>3</sup>H]-dihydroFK506.

5 The term "coated microscintillation plates" refers to streptavidin-coated microscintillation plates when the tagged ligand is biotinylated, and to anti-epitope antibody bound to anti-antibody-coated or protein A-coated microscintillation plates when the tagged ligand is epitope-tagged. Examples of coated microscintillation plates useful in the instant invention are streptavidin-coated, sheep anti-rabbit-coated, and goat anti-mouse-coated FlashPlate Plus (DuPont-NEN). Additional  
10 coatings, including but not limited to protein A, may be applied to uncoated FlashPlates by methods known to those skilled in the art.

The term "control assay" refers to the assay when performed in the presence of the tagged ligand, the fusion protein, the radiolabeled ligand and the coated microscintillation plates, but in the  
15 absence of the test compound.

The term FK506-binding proteins may include, but are not limited to, the below listed FKBP and FKBP homologues, which include a citation to the references which disclose them. This list is not  
20 intended to limit the scope of the invention.

**Mammalian**

FKBP-12	Galat et al., <i>Eur. J. Biochem.</i> , 216:689-707 (1993).
FKBP-12.6	Wiederrecht, G. and F. Etzkorn 25 <i>Perspectives in Drug Discovery and Design</i> , 2:57-84 (1994).
FKBP-13	Galat et al., <i>supra</i> ; Wiederrecht and Etzkorn, <i>supra</i> .
FKBP-25	Galat et al., <i>supra</i> ; Wiederrecht and Etzkorn, <i>supra</i> .
30 FKBP-38	Wiederrecht and Etzkorn, <i>supra</i> .
FKBP-51	Baughman et al., <i>Mol. Cell. Biol.</i> , 8, 4395-4402(1995).
FKBP-52	Galat et al., <i>supra</i> .



- 7 -

**Bacteria**

- |    |                           |   |
|----|---------------------------|---|
|    | Legionella pneumophila    | Galat et al., <i>supra</i> .  |
|    | Legionella micadei        | Galat et al., <i>supra</i> .  |
| 5  | Chlamydia trachomatis     | Galat et al., <i>supra</i> .  |
|    | E. coli fkpA              | Horne, S.M. and K.D. Young, <i>Arch. Microbiol.</i> , 163:357-365 (1995).             |
|    | E. coli slyD              | Roof et al., <i>J. Biol. Chem.</i> 269:2902-2910 (1994).                              |
| 10 | E. coli orf149            | Trandinh et al., <i>FASEB J.</i> 6:3410-3420 (1992).                                  |
|    | Neisseria meningitidis    | Hacker, J. and G. Fischer, <i>Mol. Micro.</i> , 10:445-456 (1993).                    |
|    | Streptomyces chrysomallus | Hacker and Fischer, <i>supra</i> .  |
| 15 |                           |   |
|    | <b>Fungal</b>             |   |
|    | yeast FKBP-12             | Cardenas et al., <i>Perspectives in Drug Discovery and Design</i> , 2:103-126 (1994). |
| 20 | yeast FKBP-13             | Cardenas et al., <i>supra</i> .   |
|    | yeast NPR1(FPR3)          | Cardenas et al., <i>supra</i> .   |
|    | Neurospora                | Galat et al., <i>supra</i> .  |

- 25 A variety of host cells may be used in this invention, which include, but are not limited to, bacteria, yeast, bluegreen algae, plant cells, insect cells and animal cells.

- Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors
- 30 can be used to express genes in a variety of host cells, such as, bacteria, yeast, bluegreen algae, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells. An

- 8 -

appropriately constructed expression vector may contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA  
5 sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically  
10 designed plasmids or viruses. Commercially available vectors suitable for FKBP fusion protein expression include, but are not limited to pBR322 (Promega), pGEX (Amersham), pT7 (USB), pET (Novagen), pIBI (IBI), pProEX-1 (Gibco/BRL), pBluescript II (Stratagene), pTZ18R and pTZ19R (USB), pSE420 (Invitrogen), pVL1392 (Invitrogen), pBlueBac (Invitrogen), pBacPAK (Clontech), pHIL  
15 (Invitrogen), pYES2 (Invitrogen), pCDNA (Invitrogen), pREP (Invitrogen) or the like.

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, infection, protoplast fusion, and  
20 electroporation.

*E. coli* containing an expression plasmid with the target gene fused to FKBP are grown and appropriately induced. The cells are then pelleted and resuspended in a suitable buffer. Although FKBP-12 lacks sequences that specifically direct it to the periplasm, FKBP  
25 fusions are primarily located there and can be released by a standard freeze/thaw treatment of the cell pellet. Following centrifugation, the resulting supernatant contains >80% pure FKBP fusion, which if desired can be purified further by conventional methods. Alternatively, the assay is not dependent on pure protein and the initial periplasmic  
30 preparation may be used directly. A thrombin site located between FKBP and the target protein can be used as a means to cleave FKBP from the fusion; such cleaved material may be a suitable negative control for subsequent assays.

- 9 -

A fusion protein which contains a single or multiple SH2 domain(s) may be purified by preparing an affinity matrix consisting of biotinylated phosphopeptide coupled to avidin or streptavidin immobilized on a solid support. A freeze/thaw extract is prepared from the cells which express the fusion protein and is loaded onto the affinity matrix. The desired fusion protein is then specifically eluted with phenyl phosphate.

To assay the formation of a complex between a target protein and its ligand, the tagged ligand is mixed with the FKBP fusion protein in a suitable buffer in the presence of the radiolabeled ligand. After a suitable incubation period to allow complex formation to occur, the mixture is transferred to a coated microscintillation plate to capture the tagged ligand and any bound fusion protein. The plate is sealed, incubated for a sufficient period to allow the capture to go to completion, then counted in a multiwell scintillation counter. Screening for agonists/antagonists/inhibitors is carried out by performing the initial incubation prior to the capture step in the microscintillation plate in the presence of a test compound(s) to determine whether they have an effect upon the binding of the tagged ligand to the fusion protein. This principle is illustrated in Figure 1.

The present invention can be understood further by the following examples, which do not constitute a limitation of the invention.

25

### EXAMPLE 1

#### Process for Preparing the FKBP fusion cloning vector

General techniques for modifying and expressing genes in various host cells can be found in Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. Current Protocols in Molecular Biology (John Wiley & Sons, New York, New York, 1989). Sequences for a 3'- altered FKBP fragment that contained a glycine codon (GGT) in place of the stop (TGA) codon followed by a sequence encoding a thrombin site (Leu-Val-Pro-Arg) and *Bam*HI restriction site (GAATTC) were amplified using the polymerase chain

- 10 -

- reaction (PCR). The PCR reaction contained the following primers: 5'-GATCGCCATGGGAGTGCAGGTGGAAACCATCTCCCCA-3' and 5'-TACGAATTCTGGCGTGGATCCACGCGGAACCAGACCTTCCAGTTTAG-3' and a plasmid containing human FKBP-12 as the template.
- 5 The resulting 367 base pair amplification product was ligated into the vector pCRII (Invitrogen) and the ligation mixture transformed into competent *Escherichia coli* cells. Clones containing an insert were identified using PCR with flanking vector primers. Dideoxy DNA sequencing confirmed the nucleotide sequence of one positive isolate.
- 10 The altered 338 base pair FKBP fragment was excised from the pCRII plasmid using *NcoI* and *BamHI* and ligated into *NcoI* and *BamHI* digested pET9d (Novagen) plasmid. Competent *E. coli* were transformed with the ligation mixture, and colonies containing the insert were identified using PCR with primers encoding for flanking vector
- 15 sequences. The FKBP fusion cloning vector is called pET9dFKBPt.

## EXAMPLE 2

- Process for Preparing the FK-ZAP fusion expression vector
- 20 A DNA fragment encoding for the tandem SH2 domains of ZAP-70 was prepared by PCR to contain a *BamHI* site at the 5'-end such that the reading frame was conserved with that of FKBP in the fusion vector. At the 3'-end, the fragment also incorporated a stop codon followed by a *BamHI* site. The PCR reaction contained Molt-4
- 25 cDNA (Clontech) and the following primers:  
5'-ATTAGGATCCATGCCAGATCCTGCAGCTCACCTGCCCT-3' and  
5'-ATATGGATCCTTACCAGAGGCGTTGCT-3'. The fragment was cloned into a suitable vector, sequenced, digested with *BamHI*, and the insert containing the SH2 domains ligated to *BamHI* treated
- 30 pET9dFKBPt, and transformed into *E. coli*. Clones containing inserts in the correct orientation were identified by PCR or restriction enzyme analysis. Plasmid DNA was prepared and used to transform BL21(DE3) cells.

- 11 -

EXAMPLE 3Process for Preparing the FK-SYK fusion expression vector

The expression vector for the tandem SH2 domains of Syk fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Raji cell cDNA (Clontech) and the following primers: 5'-CAATAGGATCCATGGCCAGCAGCGGCATGGCTGA-3' and 5'-GACCTAGGATCCCTAATTAACATTTCCCTGTGTGCCGAT-3'.

10

EXAMPLE 4Process for Preparing the FK-LCK fusion expression vector

The expression vector for the SH2 domain of Lck fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Molt-4 cDNA (Clontech) and the following primers:  
5'-ATATGGATCCATGGCGAACAGCCTGGAGCCCCGAACCCT-3'  
and  
5'-ATTAGGATCCTTAGGTCTGGCAGGGGCGGCTCAACCGTGTGCA-3'.

20

EXAMPLE 5FK-ZAP

25

Step A: Process for Expression of FK-ZAP

*E. coli* BL21(DE3) cells containing the pET9dFKBPt/ZapSH2 plasmid were grown in Luria-Bertani (LB) media containing 50 microgram/ml kanamycin at about 37 degrees C until the optical density measured at 600 nm was about 0.5-1.0. Expression of the FK-ZAP fusion protein was induced with 0.1 mM isopropyl beta-thiogalactopyranoside and the cells were grown for another 3-5 hr at about 30 degrees C. They were pelleted at 4400 x g for about 10 min at about 4 degrees C and resuspended in 2% of the original culture volume with 100 mM tris pH 8.0 containing 1 microgram/ml each aprotinin,

35

- 12 -

pepstatin, leupeptin, and bestatin. The resuspended pellet was frozen at about -20 degrees C until further purification.

Step B: Process for Purification of FK-ZAP

5           The affinity matrix for purification of FK-ZAP was prepared by combining agarose-immobilized avidin with excess biotinylated phosphopeptide derived from the  $\zeta$ 1 ITAM sequence of the human T-cell receptor, biotinyl-GSNQLpYNELNLGRREEpYDVLDK, and washing out unbound peptide. Frozen cells containing FK-ZAP  
10       were thawed in warm water, refrozen on dry ice for about 25 min., then thawed again. After the addition of 0.1% octyl glucoside, 1 mM dithiothreitol (DTT) and 500 mM NaCl, the extract was centrifuged at 35,000 x g for approximately 30 minutes. The supernatant was loaded onto the phosphopeptide affinity column, at about 4° and washed with  
15       phosphate buffered saline containing 1 mM DTT and 0.1% octyl glucoside. FK-ZAP was eluted with 200 mM phenyl phosphate in the same buffer at about 37°. The protein pool was concentrated and the phenyl phosphate removed on a desalting column. The purified FK-ZAP was stored at about -30° in 10 mM HEPES/150 mM NaCl/1 mM  
20       DTT/0.1 mM EDTA/10% glycerol.

EXAMPLE 6

FK-SYK

25           *E. coli* BL21(DE3) cells containing the pET9dFKBPt/SykSH2 plasmid were grown, induced, and harvested as described in Example 5. FK-SYK was purified using the same affinity matrix and methodology described in Example 5.

30           EXAMPLE 7

FK-LCK

35           *E. coli* BL21(DE3) cells containing the pET9dFKBPt/LckSH2 plasmid were grown, induced, and harvested as described in

- 13 -

Example 5. The affinity matrix for purification of FK-LCK was prepared by combining agarose-immobilized avidin with excess biotinyl- EPQpYEEIPIYL, and washing out unbound peptide. The remaining methodology for purification was the same as Example 5.

5

### EXAMPLE 8

#### Assay of phosphopeptide binding to FK-ZAP

Assays were conducted at ambient temperature in a buffer consisting of 25 mM HEPES, 10 mM DTT, 0.01% TWEEN-20, pH 7.0. 300 µl of a mixture of buffer and varying amounts of biotinyl-phosphopeptide were combined with 25 µl of FK-ZAP protein and 50 µl of [<sup>3</sup>H]-dihydroFK506 (DuPont NEN) in microfuge tubes. A 150 µl portion of each assay was then transferred to the well of a streptavidin-coated FlashPlate Plus (DuPont-NEN) and an additional 50 µl of buffer was added. Final concentrations of the assay components were:

0-50 nM biotinyl-GSNQLpYNELNLGRREEpYDVLDK  
100 nM FK-ZAP fusion protein  
25 nM [<sup>3</sup>H]-dihydroFK506

20 The plate was sealed and incubated 20 hours. Plate-bound radioactivity was measured at various timepoints in a Packard Topcount microplate scintillation counter.

### EXAMPLE 9

25

#### Method of Screening for Antagonists of FK-ZAP

Assays are conducted at ambient temperature in a buffer consisting of 25 mM HEPES, 10 mM DTT, 0.01% TWEEN-20, pH 7.0. 10 µl of a DMSO solution of test compound(s) and 120 µl of biotinyl-phosphopeptide stock solution are dispensed into the wells of a standard 96-well plate. Next, 20 µl of a mixture of FK-ZAP protein and [<sup>3</sup>H]-dihydroFK506 (DuPont NEN) are added to each test well. The assays are then transferred to the wells of a streptavidin-coated FlashPlate (DuPont NEN). Final concentrations of the assay components are:

35

- 14 -

25 nM biotinyl-GSNQLpYNELNLGRREEpYDVLDK  
25 nM FK-ZAP fusion protein  
10 nM [<sup>3</sup>H]-dihydroFK506  
5% DMSO

- 5 The plate is sealed and incubated between 1 and 8 hours. Bead-bound radioactivity is then measured in a Packard Topcount microplate scintillation counter.

#### EXAMPLE 10

10

##### Method of Screening for Antagonists of FK-SYK

The assays are conducted as set forth in Example 9, except that FK-SYK replaces FK-ZAP.

15

#### EXAMPLE 11

##### Method of Screening for Antagonists of FK-LCK

The assays are conducted as set forth in Example 9, except that FK-LCK replaces FK-ZAP and the tagged ligand is 25 nM

20 biotinyl-EPQpYEEIPIYL.



- 15 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Salowe, Scott P.  
Marcy, Alice I.  
Wisniewski, Douglas
- (ii) TITLE OF INVENTION: A High Throughput Assay Using Fusion Proteins
- (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Valerie J. Camara
  - (B) STREET: 126 E. Lincoln Avenue, P.O. Box 2000
  - (C) CITY: Rahway
  - (D) STATE: NJ
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 07065
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Camara, Valerie J.
  - (B) REGISTRATION NUMBER: 35,090
  - (C) REFERENCE/DOCKET NUMBER: 19524
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (908) 594-3902
  - (B) TELEFAX: (908) 594-4720

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1137 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- 16 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC	60
CAGACCTGCG TGGTGCACTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCTCTC	120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG	180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT	240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC	300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGCC AGATCCTGCA	360
GCTCACCTGC CCTTCTTCTA CGGCAGCATC TCGCGTGCCG AGGCCGAGGA GCACCTGAAG	420
CTGGCGGGCA TGGCGGACGG GCTCTTCCTG CTGCGCCAGT GCCTGCGCTC GCTGGGCGGC	480
TATGTGCTGT CGCTCGTGCA CGATGTGCGC TTCCACCACT TTCCCATCGA GCGCCAGCTC	540
AACGGCACCT ACGCCATTGC CGGCGGCAAA GCGCACTGTG GACCGGCAGA GCTCTGCGAG	600
TTCTACTCGC GCGACCCCGA CGGGCTGCCC TGCAACCTGC GCAAGCCGTG CAACCGGCCG	660
TCGGGCCTCG AGCCGCAGCC GGGGGTCTTC GACTGCCTGC GAGACGCCAT GGTGCGTGAC	720
TACGTGCGCC AGACGTGGAA GCTGAGGGC GAGGCCCTGG AGCAGGCCAT CATCAGCCAG	780
GCCCCGAGG TGGAGAAGCT CATTGCTACG ACGGCCACG AGCGGATGCC CTGGTACCAC	840
AGCAGCCTGA CGCGTGAGGA GGCCGAGCGT AAACCTTACT CTGGGGCGCA GACCGACGGC	900
AAGTTCCTGC TGAGGCCGCG GAAGGAGCAG GGCACATACG CCCTGTCCCT CATCTATGGG	960
AAGACGGTGT ACCACTACCT CATCAGCCAA GACAAGGCGG GCAAGTACTG CATTCCCGAG	1020
GGCACCAAGT TTGACACGCT CTGGCAGCTG GTGGAGTATC TGAAGCTGAA GGCGGACGGG	1080
CTCATCTACT GCCTGAAGGA GGCCTGCCCC AACAGCAGTG CCAGCAACGC CTCTTAA	1137

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1155 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC	60
---	----

- 17 -

CAGACCTGCG TGGTGCAC TA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCTCTCC	120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG	180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT	240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCAC CACATGCCAC TCTCGTCTTC	300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC CAGCAGCGGC	360
ATGGCTGACA GCGCCAACCA CCTGCCCTTC TTTTTCGGCA ACATCACCCG GGAGGAGGCA	420
GAAGATTACC TGGTCCAGGG GGGCATGAGT GATGGGCTTT ATTTGCTGCG CCAGAGCCGC	480
AACTACCTGG GTGGCTTCGC CCTGTCCGTG GCCCACGGGA GGAAGGCACA CCACTACACC	540
ATCGAGCGGG AGCTGAATGG CACCTACGCC ATCGCCGGTG GCAGGACCCA TGCCAGCCCC	600
GCCGACCTCT GCCACTACCA CTCCCAGGAG TCTGATGGCC TGGTCTGCCT CCTCAAGAAG	660
CCCTTCAACC GGCCCCAAGG GGTGCAGCCC AAGACTGGGC CCTTTGAGGA TTTGAAGGAA	720
AACCTCATCA GGAATATGT GAAGCAGACA TGGAACCTGC AGGGTCAGGC TCTGGAGCAG	780
GCCATCATCA GTCAGAAGCC TCAGCTGGAG AAGCTGATCG CTACCACAGC CCATGAAAAA	840
ATGCCTTGGT TCCATGGAAA AATCTCTCGG GAAGAATCTG AGCAAATTGT CCTGATAGGA	900
TCAAAGACAA ATGGAAAGTT CCTGATCCGA GCCAGAGACA ACAACGGCTC CTACGCCCTG	960
TGCCTGCTGC ACCAAGGGAA GGTGCTGCAC TATCGCATCG ACAAAGACAA GACAGGGAAG	1020
CTCTCCATCC CCGAGGGAAA GAAGTTCGAC ACGCTCTGGC AGCTAGTCGA GCATTATTCT	1080
TATAAAGCAG ATGGTTTGTT AAGAGTTCTT ACTGTCCCAT GTCAAAAAAT CGGCACACAG	1140
GGAAATGTTA ATTAG	1155

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 675 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC	60
---	----

- 18 -

CAGACCTGCG TGGTGCAC TA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC 120  
 CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180  
 GAAGAAGGGG TTGCCCAGAT GACTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240  
 TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300  
 GATGTGGAGC TTCTAAACT GGAAGGTCTG GPTCCGCGTG GATCCATGGC GAACAGCCTG 360  
 GAGCCCGAAC CCTGGTCTT CAAGAACCTG AGCCGCAAGG ACGCGGAGCG GCAGCTCCTG 420  
 GCGCCCGGGA AACTCACGG CTCTTCCTC ATCCGGGAGA GCGAGAGCAC CGCGGGATCG 480  
 TTTTCACTGT CGGTCCGGA CTTCGACCAG AACCAGGGAG AGGTGGTGAA ACATTACAAG 540  
 ATCCGTAATC TGGACAACGG TGGCTTCTAC ATCTCCCTC GAATCACTTT TCCCGGCCTG 600  
 CATGAACTGG TCCGCCATTA CACCAATGCT TCAGATGGGC TGTGCACACG GTTGAGCCGC 660  
 CCCTGCCAGA CCTAA 675

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 378 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe  
 1 5 10 15  
 Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu  
 20 25 30  
 Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys  
 35 40 45  
 Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val  
 50 55 60  
 Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp  
 65 70 75 80  
 Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala  
 85 90 95

- 19 -

Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro  
 100 105 110  
 Arg Gly Ser Met Pro Asp Pro Ala Ala His Leu Pro Phe Phe Tyr Gly  
 115 120 125  
 Ser Ile Ser Arg Ala Glu Ala Glu Glu His Leu Lys Leu Ala Gly Met  
 130 135 140  
 Ala Asp Gly Leu Phe Leu Leu Arg Gln Cys Leu Arg Ser Leu Gly Gly  
 145 150 155 160  
 Tyr Val Leu Ser Leu Val His Asp Val Arg Phe His His Phe Pro Ile  
 165 170 175  
 Glu Arg Gln Leu Asn Gly Thr Tyr Ala Ile Ala Gly Gly Lys Ala His  
 180 185 190  
 Cys Gly Pro Ala Glu Leu Cys Glu Phe Tyr Ser Arg Asp Pro Asp Gly  
 195 200 205  
 Leu Pro Cys Asn Leu Arg Lys Pro Cys Asn Arg Pro Ser Gly Leu Glu  
 210 215 220  
 Pro Gln Pro Gly Val Phe Asp Cys Leu Arg Asp Ala Met Val Arg Asp  
 225 230 235 240  
 Tyr Val Arg Gln Thr Trp Lys Leu Glu Gly Glu Ala Leu Glu Gln Ala  
 245 250 255  
 Ile Ile Ser Gln Ala Pro Gln Val Glu Lys Leu Ile Ala Thr Thr Ala  
 260 265 270  
 His Glu Arg Met Pro Trp Tyr His Ser Ser Leu Thr Arg Glu Glu Ala  
 275 280 285  
 Glu Arg Lys Leu Tyr Ser Gly Ala Gln Thr Asp Gly Lys Phe Leu Leu  
 290 295 300  
 Arg Pro Arg Lys Glu Gln Gly Thr Tyr Ala Leu Ser Leu Ile Tyr Gly  
 305 310 315 320  
 Lys Thr Val Tyr His Tyr Leu Ile Ser Gln Asp Lys Ala Gly Lys Tyr  
 325 330 335  
 Cys Ile Pro Glu Gly Thr Lys Phe Asp Thr Leu Trp Gln Leu Val Glu  
 340 345 350  
 Tyr Leu Lys Leu Lys Ala Asp Gly Leu Ile Tyr Cys Leu Lys Glu Ala  
 355 360 365  
 Cys Pro Asn Ser Ser Ala Ser Asn Ala Ser  
 370 375

(2) INFORMATION FOR SEQ ID NO:5:

- 20 -

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1             5             10             15
Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
 20             25             30
Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
 35             40             45
Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
 50             55             60
Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
 65             70             75             80
Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
 85             90             95
Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
100            105            110
Arg Gly Ser Met Ala Ser Ser Gly Met Ala Asp Ser Ala Asn His Leu
115            120            125
Pro Phe Phe Phe Gly Asn Ile Thr Arg Glu Glu Ala Glu Asp Tyr Leu
130            135            140
Val Gln Gly Gly Met Ser Asp Gly Leu Tyr Leu Leu Arg Gln Ser Arg
145            150            155            160
Asn Tyr Leu Gly Gly Phe Ala Leu Ser Val Ala His Gly Arg Lys Ala
165            170            175
His His Tyr Thr Ile Glu Arg Glu Leu Asn Gly Thr Tyr Ala Ile Ala
180            185            190
Gly Gly Arg Thr His Ala Ser Pro Ala Asp Leu Cys His Tyr His Ser
195            200            205
Gln Glu Ser Asp Gly Leu Val Cys Leu Leu Lys Lys Pro Phe Asn Arg
210            215            220

```

- 21 -

```

Pro Gln Gly Val Gln Pro Lys Thr Gly Pro Phe Glu Asp Leu Lys Glu
225                230                235                240

Asn Leu Ile Arg Glu Tyr Val Lys Gln Thr Trp Asn Leu Gln Gly Gln
                245                250                255

Ala Leu Glu Gln Ala Ile Ile Ser Gln Lys Pro Gln Leu Glu Lys Leu
                260                265                270

Ile Ala Thr Thr Ala His Glu Lys Met Pro Trp Phe His Gly Lys Ile
                275                280                285

Ser Arg Glu Glu Ser Glu Gln Ile Val Leu Ile Gly Ser Lys Thr Asn
                290                295                300

Gly Lys Phe Leu Ile Arg Ala Arg Asp Asn Asn Gly Ser Tyr Ala Leu
305                310                315                320

Cys Leu Leu His Glu Gly Lys Val Leu His Tyr Arg Ile Asp Lys Asp
                325                330                335

Lys Thr Gly Lys Leu Ser Ile Pro Glu Gly Lys Lys Phe Asp Thr Leu
                340                345                350

Trp Gln Leu Val Glu His Tyr Ser Tyr Lys Ala Asp Gly Leu Leu Arg
                355                360                365

Val Leu Thr Val Pro Cys Gln Lys Ile Gly Thr Gln Gly Asn Val Asn
370                375                380

```

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 224 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
1                5                10                15

Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
                20                25                30

Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
35                40                45

```

- 22 -

Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val  
 50 55 60  
 Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp  
 65 70 75 80  
 Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala  
 85 90 95  
 Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro  
 100 105 110  
 Arg Gly Ser Met Ala Asn Ser Leu Glu Pro Glu Pro Trp Phe Phe Lys  
 115 120 125  
 Asn Leu Ser Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro Gly Asn  
 130 135 140  
 Thr His Gly Ser Phe Leu Ile Arg Glu Ser Glu Ser Thr Ala Gly Ser  
 145 150 155 160  
 Phe Ser Leu Ser Val Arg Asp Phe Asp Gln Asn Gln Gly Glu Val Val  
 165 170 175  
 Lys His Tyr Lys Ile Arg Asn Leu Asp Asn Gly Gly Phe Tyr Ile Ser  
 180 185 190  
 Pro Arg Ile Thr Phe Pro Gly Leu His Glu Leu Val Arg His Tyr Thr  
 195 200 205  
 Asn Ala Ser Asp Gly Leu Cys Thr Arg Leu Ser Arg Pro Cys Gln Thr  
 210 215 220



- 23 -

WHAT IS CLAIMED IS:

1. A method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

- 5       a) mixing a test compound, a tagged ligand, the fusion protein, and a radiolabeled ligand;
- b) adding the mixture to a coated microscintillation plate;
- c) incubating the mixture for between about 1 hour and about 24 hours;
- 10       d) measuring the plate-bound counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound using scintillation counting; and
- e) determining the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.
- 15

2. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 1, wherein the tagged ligand is a biotinylated ligand or epitope-tagged ligand.

20

3. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 2, wherein the coated microscintillation plates are streptavidin-coated or anti-antibody or protein A-coated.

25

4. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 3, wherein the radiolabeled ligand consists of a [<sup>3</sup>H]-, [<sup>125</sup>I]-, [<sup>14</sup>C]-, [<sup>35</sup>S]-, [<sup>32</sup>P]-, or [<sup>33</sup>P]-labeled FK506 analog.

30

5. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 4, wherein the fusion protein comprises an FK506-binding protein linked through a peptide linker to a target protein.

- 24 -

6. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 5, wherein the target protein comprises a single or multiple signal transduction domain.

5

7. The method for screening for compounds capable of binding to a fusion protein, as recited in Claim 6, wherein the single or multiple signal transduction domain is selected from the group consisting of: SH1, SH2, SH3 and PH domains.

10

8. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 7, wherein the target protein is a single or multiple SH2 domain.

15

9. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 8, wherein the radiolabeled ligand is [<sup>3</sup>H]-dihydroFK506.

20

10. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 9, wherein the FK506-binding protein is a 12kDA human FK506-binding protein.

25

11. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 10, wherein the target protein is a single or multiple SH2 domain selected from the group consisting of: ZAP:SH2, SYK:SH2 and LCK:SH2.

30

12. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, ZAP:SH2.

13. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, SYK:SH2.

- 25 -

14. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, LCK:SH2.

5

15. A process for preparing a recombinant DNA expression vector encoding for a fusion protein comprising the steps of:

- a) removing the stop codon on DNA encoding for an FK506-binding protein;
- 10 b) synthesizing a modified DNA fragment on the DNA encoding for the FK506-binding protein which encodes for a peptide linker;
- c) digesting an expression vector at cloning sites;
- d) cloning the modified DNA fragment encoding for the FK506-binding protein with a peptide linker into the digested expression vector to generate a recombinant DNA expression vector encoding for FK506-binding protein with a peptide linker; and
- 15 e) cloning DNA encoding for a target protein into a recombinant DNA expression vector encoding for FK506-binding protein with a peptide linker to produce the recombinant DNA expression vector encoding for the fusion protein.

20

16. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 15, wherein the target protein is a single or multiple signal transduction domain.

25

17. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 16, wherein the single or multiple signal transduction domain is selected from the group consisting of: SH1, SH2, SH3 and PH domains.

30

- 26 -

18. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 17, wherein the single or multiple signal transduction domain is an SH2 domain.

5

19. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 18, wherein the single or multiple signal transduction domain is an SH2 domain selected from the group consisting of ZAP:SH2, SYK:SH2 and LCK:SH2.

10

20. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 19, wherein the FK506-binding protein is a 12 kDa FK506 binding protein.

15

21. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 20, wherein the peptide linker has the amino acid sequence GLVPRGS.

20

22. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 21, wherein the expression vector is selected from the group consisting of: pBR322, pGEX, pT7, pET, pIBI, pProEX-1, pBluescript II, pTZ18R and pTZ19R, pSE420, pVL1392, pBlueBac, pBacPAK, pHIL, pYES2, pCDNA, and pREP.

25

23. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 22, wherein the expression vector is the T7 RNA polymerase based pET expression vector.

30

24. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 23, wherein the target protein is ZAP:SH2.

- 27 -

25. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 23, wherein the target protein is SYK:SH2.
- 5
26. The process for preparing a recombinant DNA expression vector encoding the fusion protein, as recited in Claim 23, wherein the target protein is LCK:SH2.
- 10
27. Isolated DNA encoding for a fusion protein comprising the sequence:  
(SEQ. ID. NO. 1).
- 15
28. Isolated DNA encoding for a fusion protein comprising the sequence:  
(SEQ. ID. NO. 2).
- 20
29. Isolated DNA encoding for a fusion protein comprising the sequence:  
(SEQ. ID. NO. 3).
- 25
30. A FKBP-ZAP:SH2 fusion protein comprising the sequence:  
(SEQ. ID. NO. 4).
- 30
31. A FKBP-SYK:SH2 fusion protein comprising the sequence:  
(SEQ. ID. NO. 5).
32. A FKBP-LCK:SH2 fusion protein comprising the sequence:  
(SEQ. ID. NO. 6).

- 28 -

33. A process for expressing recombinant DNA encoding for a fusion protein in an expression vector comprising the steps of:

- a) transforming a host cell with the fusion protein expression vector;
- 5 b) inducing expression of the fusion protein in the host cell;
- c) recovering the fusion protein from the host cell; and
- d) purifying the fusion protein.

34. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 33, wherein the target  
10 protein is a single or multiple signal transduction domain.

35. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 34, wherein the single or  
15 multiple signal transduction domain is selected from the group consisting of: SH1, SH2, SH3 and PH domains.

36. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 35, wherein the single or  
20 multiple signal transduction domain is a single or multiple SH2 domain.

37. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 36, wherein the single or  
25 multiple SH2 domain is selected from a group consisting of ZAP:SH2, SYK:SH2 and LCK:SH2.

38. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 37, wherein the FK506-  
30 binding protein is human 12kDa FK506-binding protein.

39. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 38, wherein the host cell  
is from bacteria, yeast, blue green algae, plant cells, insect cells, or  
animal cells.

- 29 -

40. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 39, wherein the expression vector is T7 RNA polymerase based expression vector.

5

41. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 40, wherein the host cell is an *E. coli* strain selected from a group consisting of BL21 (DE3), Nova Blue (DE3), and JM109 (DE3).

10

42. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 41, wherein the single or multiple SH2 domain is ZAP:SH2.

15

43. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 41, wherein the single or multiple SH2 domain is SYK:SH2.

20

44. The process for expressing recombinant DNA encoding a fusion protein, as recited in Claim 41, wherein the single or multiple SH2 domain is LCK:SH2.

45. The process for purifying an isolated FKBP-SH2 fusion protein comprising the steps of:

25

a) preparing an affinity matrix consisting of biotinylated phosphopeptide coupled to avidin or streptavidin immobilized on a solid support;

b) preparing a freeze/thaw extract from cells expressing the fusion protein;

30

c) loading the extract onto the affinity matrix and washing off unbound protein; and

d) eluting the desired fusion protein with phenyl phosphate.

- 30 -

46. A recombinant FKBP-SH2 domain T7 RNA polymerase-based expression vector, wherein the DNA encodes for the FKBP-ZAP:SH2 fusion protein and has the DNA sequence (SEQ. ID. NO. 1).

5

47. A recombinant FKBP-SH2 domain T7 RNA polymerase-based expression vector, wherein the DNA encodes for the FKBP-SYK:SH2 fusion protein and has the DNA sequence (SEQ. ID. NO. 2).

10

48. A recombinant FKBP-SH2 domain T7 RNA polymerase-based expression vector, wherein the DNA encodes for the FKBP-LCK:SH2 fusion protein and has the DNA sequence (SEQ. ID. NO. 3).

15

49. A recombinant host cell containing the recombinant FKBP-SH2 domain T7 RNA polymerase-based expression vector wherein the recombinant host cell is selected from the group consisting of: *E. coli* BL21 (DE3), *E. coli* Nova Blue (DE3), and *E. coli* JM109 (DE3).

20

50. The recombinant host cell containing the recombinant FKBP-SH2 domain T7 RNA polymerase-based expression vector as recited in claim 49, wherein the recombinant host cell is *E. coli* BL21 (DE3).

25



1/1

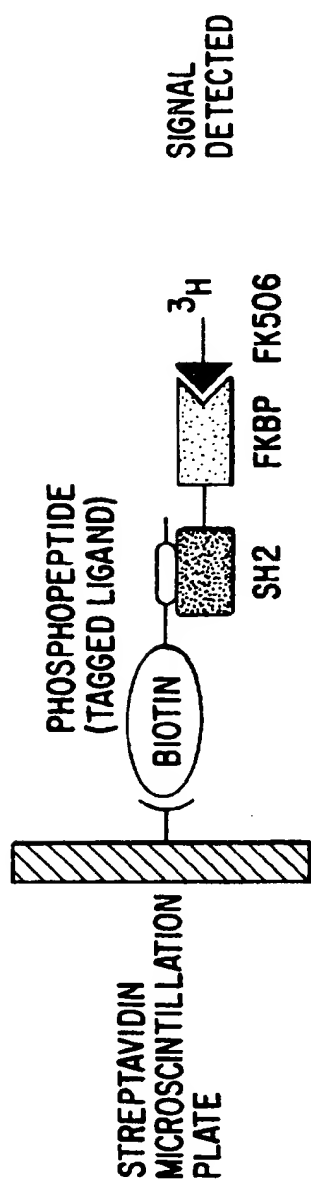


FIG. 1A

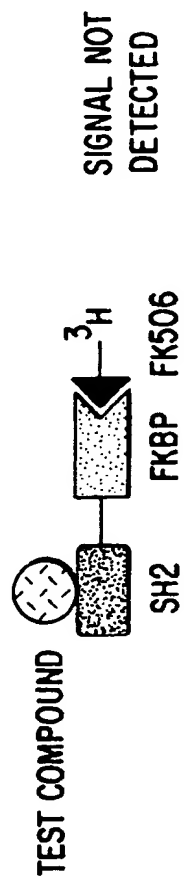


FIG. 1B

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/14567**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.7, 70.1, 70.3, 71.1, 71.2, 172.3, 252.33, 320.1; 436/501; 530/350, 413; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P --- Y,P	US 5,498,597 A (BURAKOFF et al.) 12 March 1996, column 12, lines 28-67.	15 ----- 1-14, 16-50
Y,P	US 5,464,745 A (MIERENDORF et al.) 07 November 1995, column 7, lines 36-42.	1-14
A	US 5,434,064 A (SCHLESSINGER et al.) 18 July 1995.	1-50
Y,E	US 5,580,979 A (BACHOVCHIN) 03 December 1996, column 4, lines 10-19.	11-14, 24-32, 37-44, 46-50
X -- Y	US 5,352,660 A (PAWSON) 04 October 1994, column 8, lines 29-58; column 9, lines 15-26; column 10, lines 39-42; column 11, lines 11-20.	33-36 ----- 1-14

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

13 DECEMBER 1996

Date of mailing of the international search report

31 JAN 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

BRIAN K. LATHROP

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/14567

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,534,424 A (UHLEN et al.) 09 July 1996, Fig. 1.	2-14
Y	STUDIER et al. Use of T7 RNA polymerase to direct expression of cloned genes. Methods in Enzymology. 1990, Vol. 185, pages 60-89, the entire document.	22-32, 39-44, 46-50
A,P	SONATORE et al. The utility of FK506-binding protein as a fusion partner in scintillation proximity assays: application to SH2 domains. Analytical Biochemistry. 05 September 1996, Vol. 240, No. 2, pages 289-297.	
X	GILMER et al. Peptide inhibitors of src SH3-SH2- phosphoprotein interactions. The Journal of Biological Chemistry. 16 December 1994, Vol. 269, No. 50, pages 31711-31719, the entire document.	33-36
Y		1-32, 37-50
A,P	MULLER et al. Rapid identification of phosphopeptide ligands for SH2 domains: screening of peptide libraries by fluorescence-activated bead sorting. The Journal of Biological Chemistry. 12 July 1996, Vol. 271, No. 28, pages 16500-16505.	1-50

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/14567

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 21/04; C07K 14/00, 14/195, 14/435, 17/00, 17/06, 17/14; C12N 1/21, 15/00, 15/09, 15/63, 15/70; C12P 21/00, 21/02; G01N 33/53, 33/547, 33/566; A23J 1/00

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/69.7, 70.1, 70.3, 71.1, 71.2, 172.3, 252.33, 320.1; 436/501; 530/350, 413; 536/23.4

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

USPAT, WPIDS, INPADOC, MEDLINE, SCISEARCH, CAPLUS, EMBASE

search terms: FKBP, SH2, fusion, LCK, ZAP, SYK, streptavidin, biotin, screen###, coat###, scintillation, Marcy, A., Salowe, S., Wisniewski, D.

This Page is inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record

## BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ BLACK BORDERS

☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

☒ FADED TEXT OR DRAWING

☒ BLURED OR ILLEGIBLE TEXT OR DRAWING

☐ SKEWED/SLANTED IMAGES

☐ COLORED OR BLACK AND WHITE PHOTOGRAPHS

☐ GRAY SCALE DOCUMENTS

☐ LINES OR MARKS ON ORIGINAL DOCUMENT

☐ REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images  
problems checked, please do not report the  
problems to the IFW Image Problem Mailbox**